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The NS5a Gene of Hepatitis C Virus in Patients Treated With Interferon-α

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Patients infected with hepatitis C virus (HCV) genotype 3 have a better response to interferon-α (IFN-α) therapy than those infected with genotype 1. There are extensive sequence differences between genotypes in the 3′ half of the NS5a gene. An association between IFN-α response and the interferon sensitivity-determining region (ISDR) (amino acids 2209-2248) of HCV genotype 1b has been described [Enomoto et al. (1996) New England Journal of Medicine 334: 771–776]. A prospective study was conducted to determine whether the derived NS5A amino acid sequence or quasi-species diversity could predict response to IFN-α therapy. Serum samples were obtained before, during, and after treatment from 35 IFN-α-treated patients chronically infected with HCV (eight with type1b,13 with type1a, and 14 with type3a). Nucleotide sequences were determined, and amino acid sequences corresponding to residues 2178-2390 of the polyprotein were derived. Quasi-species complexity was analysed by amplification of the ISDR region (2270-2403), followed by single-stranded conformation polymorphism (SSCP). No amino acid sequence that could be used to predict response to treatment was found, and there was no selection of specific amino acid residues during treatment. A striking lack of variability was seen in HCV genotype 3a, but the small degree of variation could suggest an effect on response. SSCP showed that variation in the predominant NS5a sequence occurred in the presence and absence of therapeutically administered IFN-α. HCV quasi-species diversity pretreatment did not predict IFN-α treatment outcome. The conclusion of the study is that the amino acid sequence of NS5a cannot be used to predict the efficacy of treatment with IFN-α in HCV-infected patients in Scotland. No evidence was found to support the selection of IFN-α-resistant strains in the NS5a gene. ***J. Med. Virol. 60:367–378, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus; response to interferon-α therapy; nucleotide sequencing; single-stranded conformation polymorphism

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease. Most individuals who are infected become long-term carriers of the virus, with the attendant risks of development of cirrhosis and hepatocellular carcinoma. Interferon-α (IFN-α) is successful in clearing the virus in only a small proportion of patients. The response to IFN-α in a given patient is probably the result of the interaction of many variables, but evidence has accumulated that patients infected with genotype 1 HCV are less likely to clear virus than are those infected with other types [Davis and Lau, 1997]. In Glasgow it was found that HCV isolates from patients were distributed roughly equally between genotypes 1 and 3, with a small number having genotype 2. It was reasoned that study of regions of the genome that differed markedly between genotypes might give clues as to how the corresponding proteins could interact differently with cellular proteins induced by IFN-α. The NS5a gene contains a variable region (V3) [Inchauspe et al., 1991] and several genotype-specific insertions and deletions [Chamberlain et al., 1997]. The protein is phosphorylated differentially [Tanji et al., 1995; Reed et al., 1997] and contains a potential nuclear localisation signal [Ide et al., 1996], both of which suggest mechanisms by which the polypeptide might be able to interfere with the response of the infected hepatocyte to IFN-α. A prospective study was initiated to sequence the 3′ end of the NS5a gene from patients before and after 12 weeks of treatment, since any selection of strains able to induce interferon resistance would have occurred by then.

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After the study had started, a group from Japan [Enomoto et al., 1995,1996] reported a 40–amino acid (aa) interferon sensitivity-determining region (ISDR) between aa 2209 and 2248 [Kato et al., 1990] in the NS5a gene of genotype 1b. All patients with the ISDR-derived aa sequence identical to that of the full-length published sequence HCV-J, referred to as the "wild type," were nonresponders (NRs). All those with four or more differences, the "mutant type," responded to treatment. Fewer than 20% with one to three aa differences ("intermediate type") responded. The current study was therefore extended to include the ISDR.

Evidence for a plausible mechanism of action by which NS5a could counteract the effects of IFN-α on infected cells has been reported [Gale et al., 1997, 1998]. It was shown that the NS5a of genotypes 1a and 1b expressed in yeast and mammalian cells could be potent inhibitors of the double-stranded RNA-dependent kinase (PKR) induced by IFN-α. Deletion studies showed that the ISDR was important in the effect observed and that 26 aa downstream of the ISDR also interact with PKR. If changes in aa sequences reported in mutant types of HCV are introduced to the constructs used in these assays, this inhibition does not occur. Using different techniques, however, a Japanese group found that deletion of the 40-aa ISDR failed to abrogate the interaction of NS5a with PKR, suggesting that additional aa play a part in the PKR–NS5a interaction [Satoh et al., 1998].

MATERIALS AND METHODS

Patients, Treatment, and Samples

Patients under 70 years of age were offered treatment with IFN-α if HCV RNA was detectable in the serum and if liver biopsy showed changes typical of chronic HCV infection. Exclusion criteria were the presence of decompensated cirrhosis; marked obesity; human immunodeficiency virus infection; other cause of chronic hepatitis, such as alcohol excess or autoimmune disease; or significant co-morbidity, for example, neoplasia, pregnancy or lactation, or intravenous drug use within the past year. Interferon alpha-2a (Roferon; Roche Products Ltd., England) was given at a dose of six million units (MU) thrice weekly. Patients with negative results for HCV by reverse transcription–polymerase chain reaction (RT-PCR) after 12 weeks continued on 3 MU thrice weekly for a further 36 weeks (total dose 540 MU), whereas those with PCR-positive results discontinued treatment. Serum samples were collected before treatment and after 12, 48, and 72 weeks; separated within 4 hours of venipuncture; and stored in aliquots at –20°C or –70°C. Permission for the study was given by the ethical committee of the West Glasgow Hospitals University NHS Trust, and informed consent was obtained from all patients.

Definition of Response

Patients who showed positive results for HCV RNA by RT-PCR at 12 weeks were categorised as NRs. Complete sustained responders (CSRs) showed negative results for HCV RNA at 48 and 72 weeks. Breakthrough (BT) was seen when the patient's results were HCV RNA negative at 12 weeks but HCV RNA positive by 48 weeks. Relapsers (Rels) showed negative results for HCV RNA at 48 weeks but positive results by 72 weeks.

Liver Histology

Percutaneous needle liver biopsies were carried out under ultrasonography guidance before and after 48 weeks of IFN-α therapy. Biopsies were stained with haematoxylin and eosin and van Gieson stains. One pathologist (Professor R. McSween) reviewed all the biopsy samples and confirmed features of chronic HCV. Biopsy samples were graded for intensity of necroinflammatory activity and scored for the severity of fibrosis using the Ishak modified histology activity index [Ishak et al., 1995].

Detection of Serum Hepatitis C Virus RNA

HCV RNA was detected by in-house nested, tagged RT-PCR or by Amplicor (Roche Products). The two PCR methods were of equal sensitivity and detected 2,000 copies of RNA per millilitre. For the in-house method, RNA was extracted from serum using the QIAamp Viral RNA Kit (QIAGEN, England) per the manufacturer's instructions. One hundred units of M-MLV RT (Gibco BRL Ltd., Scotland) was used to synthesise complementary DNA (cDNA) from 3 μL of extracted RNA. The reaction was performed in 50 mmol/L Tris-HCl (pH 8.3); 75 mmol/L KCl; 3 mmol/L $MgCl_2$; 10 mmol/L DTT; 1 mmol/L each of dGTP, dATP, dTTP, and dCTP; 40 pmol/L of primer VTAGRT (5′-TGAGATGACAGTTCATCGATTTTTCTTTGAGGTTTAGGA -3′); and 1 U RNAsin (Promega, UK) in a final volume of 20 μL. Incubation was at 25°C for 10 min, 37°C for 55 min, and 95°C for 5 min. Twenty-five cycles at 94°C for 30 sec, 55°C for 40 sec, and 70°C for 2.5 min, preceded by an incubation at 94°C for 4 min, were performed with *Taq* DNA polymerase (Gibco BRL), according to the manufacturer's instructions, on 4 μL cDNA in a final volume of 20 μL, using primers VTAG 940 (5′-GTGGTCAAGACGCCTAGAGCTTCACGCAGAAAGCGTCTAG-3′) and VTAG 1 (5′- CTGAGATGACAGTTCATCGA-3′). One microlitre of first-round PCR product was reamplified over 25 cycles as described, omitting the preliminary 94°C incubation for 4 min with primers VTAG 3 (5′-GTGGTCAAGAGCCTAGAGC-3′) and NCR4 (5′-CACTCTCGAGACCCTATCAGGCAGT-3′). PCR products were subjected to electrophoresis on 2% agarose gels containing ethidium bromide and gels visualised under ultraviolet light and photographed. Precautions to avoid contamination were adopted throughout all PCR experiments [Kwok and Higuchi, 1989].

HCV Genotyping

HCV genotype was determined by restriction fragment length polymorphism (RFLP), using a modification of a previously described method [Davidson et al.,

1995]. Briefly, 7 μL of the nested 5′NCR PCR product was digested with 2 U each of *Hae*III and *Rsa*I, *Scr*fI and *Hin*fI, *Mva*I and *Hin*fI (New England Biolabs, England) in the presence of the appropriate buffer in a final volume of 20 μL for 2 hr at 37°C. The DNA fragments were electrophoresed on a high-resolution 4% Metaphor agarose gel (FMC Bioproducts, Rockland, ME) containing 5 μg/mL ethidium bromide at 100 V for 2 hr. The resulting cleavage patterns were compared with those of reference genotypes.

Viral Load Measurement

Quantification of HCV RNA pretreatment was done by the branched DNA assay (Quantiplex HCV RNA 2.0 assay; Chiron Corp, Berkeley, CA). All serum samples had been stored at –20°C without thawing since collection. Serum samples were analysed in duplicate per the manufacturer's instructions. The quantity of HCV RNA in each sample was expressed in HCV RNA genome equivalents per millilitre (GE/mL).

Reverse Transcription–Polymerase Chain Reaction of NS5a

The part of the NS5a gene studied was amplified in two fragments. First, RNA extracted from serum was reverse-transcribed, as described previously, with Empol 4 (5′-GGCTGGATCCAGAAAACCTC-3′), and nested PCR was performed with primers 1aSen (5′-TGGA$^{T}/_{C}$GGGGTGCGCCTACATAGGT-3′) and 1aAS (5′-TAACCTCCTTGAG$^{T}/_{C}$ACGTCCTGGTA-3′), followed by 5a6 (5′-CC$^{T}/_{A}$TGCGAGCCCGAACCGGA-3′) and SP12 (5′-TT$^{G}/_{A}$TAGTCCGGCCG$^{C}/_{T}$GCCCA-3′) for type 1 strains, and Enom3Sen (5′-CACCG$^{C}/_{T}$TACGCCCCTCCATGTA -3′) with JM1 (5′-GAGTATGACAT$^{G}/_{T}$GAGCAGCA-3′), followed by 35a6 (5′-CCCTGTGAGCC$^{A}/_{C}$GAACCGGA-3′) with SP3 (5′-TTGTAGTCCGGTCTAGCCCA-3′) for type 3 strains. To obtain the second fragment, RNA extracted from serum was reverse-transcribed, as described previously, with Empol 4 (5′-GGCTGGATCCAGAAAACCTC-3′), and nested PCR was performed with Empol 21 (5′-CTGAGCTCATAGAGGCCAACCTCCT-3′) and Empol 4 followed by Empol 3 (5′-CAGGATCCGGGCGG$^{C}/_{G}$AACATCACC-3′) and JM1 for genotype 1 and JME21 (5′-GCTGAGCTAGTGGA$^{C}/_{G}$GCC AAC-3′) and Empol 4 followed by JME3 (5′-GCAGGAGATGGGC-$^{A}/_{G}$GCAACAT-3′) and JM1 for genotype 3. First-round PCR was two cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min, followed by 28 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. Second-round PCR was 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

Nucleotide Sequencing

PCR products (80 μL) were subjected to electrophoresis on 2% low-melting-point agarose gels (NuSieve; FMC Bioproducts), and DNA was extracted from the gel slices using silica gel columns (Recovery DNA purification kit II; Hybaid, England), originally using manual sequencing and more recently using an ABI Prism 377 automated sequencer (Perkin Elmer, England). Sequencing was undertaken with appropriate sense and antisense inner PCR primers. RNA was extracted and amplified from two separate aliquots of each serum, to detect any artefacts that might arise from misincorporation of bases by *Taq* polymerase.

Single-stranded Conformation Polymorphism Analysis

PCR was carried out as described for 20 cycles, and the second-round reaction volume was increased to 100 μL. Eighty microlitres of the product was subjected to electrophoresis on a 2% NuSieve gel (FMC Bioproducts), and the DNA was recovered by phenol chloroform extraction and precipitation with 3 mol/L sodium acetate and 100% ethanol on dry ice. After centrifugation, the dried DNA pellet was dissolved in 40 μL deionised water. Polymerase reactions using 1 μL DNA were performed using cycling conditions identical to PCR, except that only a single oligonucleotide was present (5a6 for type 1 and 35a6 for type 3 isolates) and 0.2 μCi [α-^{32}P]dATP was added. In some cases, single-stranded products of both senses were generated in separate reactions. The concentration of ssDNA was estimated by comparison with lambda DNA standards (1–5 ng/αL) on a 2% agarose gel.

Twenty-five nanograms of ssDNA in a final volume of 50 μL, with loading solution (0.05% xylene cyanol, 20 mmol/L EDTA, 95% formamide), was subjected to nondenaturing polyacrylamide gel electrophoresis (0.5 × MDE) (FMC Bioproducts) in 0.6 × Tris-borate-EDTA buffer at 200 V for 18 hr at room temperature. Molecular weight markers (marker VI (Boehringer Mannheim, UK) and a 123-bp ladder) and a reference patient sample were run on each gel to test reproducibility. SSCP distinguishes DNA according to conformation, not molecular weight, so the markers do not indicate the size of sample fragments. The gel was stained in 1 μg/ml ethidium bromide for 15 min, inspected under ultraviolet light, photographed, dried for 1–2 hr, and exposed to X-OMAT film (Kodak, England) at room temperature. All experiments were performed at least twice on different days on PCR products generated from separate aliquots of serum. All bands were counted independently by two observers (V.M.M., E.A.B.M.).

Computer and Statitistical Analyses

Nucleotide and aa sequences were derived and aligned using TRANSLATE, PILEUP, PRETTY, and DIVERGE programs in the Genetic Computer Group sequence analysis package. Statistical analysis was carried out using chi-square and Mann-Whitney tests and one-way analysis of variance. Logarithmic transformation of viral load, alanine aminotransferase (ALT), and age was performed to correct for strong positive skew in these variables.

TABLE 1. Patients' Pretreatment Characteristics and Responses to Inferferon*

Patient	Sex	Age (yrs)	Genotype	Viral load (10^6 GE/mL)	ALT (U/L)	Ishak score		No. aa differences from consensus	Number of variants	Interferon response[a]
						Inflammation	Fibrosis			
1	M	25	1b	1.38	470	4	1	0	1	NR
2	F	40	1b	1.55	59	N/D	N/D	2	5	NR
3	M	56	1b	9.50	69	9	6	12	1	NR
4	M	58	1b	4.10	127	7	4	5	4	NR
5	F	45	1b	5.26	109	8	5	0	2	NR
6	F	44	1b	0.00	42	2	1	1	2	REL
7	F	45	1b	1.40	370	10	3	2	5	CSR
8	M	49	1b	0.00	151	10	2	5	4	CSR
9	M	46	3a	21.43	150	3	1	0	2	NR
10	F	39	3a	0.00	63	7	1	0	2	NR
11	F	31	3a	11.95	65	7	2	0	3	BT
12	F	48	3a	11.24	112	7	5	0	1	BT
13	M	36	3a	3.08	67	8	2	0	4	BT
14	M	35	3a	7.90	56	5	1	0	2	REL
15	M	35	3a	1.47	94	7	2	0	1	REL
16	M	36	3a	39.98	178	5	1	0	2	REL
17	F	32	3a	0.78	86	6	2	0	4	REL
18	M	34	3a	0.55	74	6	3	4	3	REL
19	M	29	3a	1.66	112	5	2	0	3	CSR
20	F	27	3a	1.15	45	4	1	0	3	CSR
21	M	39	3a	1.30	120	6	1	0	1	CSR
22	M	41	3a	0.00	80	6	3	3	3	CSR
23	F	37	1a	0.23	97	4	1	3	2	NR
24	M	39	1a	1.41	69	4	1	2	2	NR
25	F	37	1a	1.23	28	4	1	2	2	NR
26	M	30	1a	1.56	148	7	1	0	4	NR
27	F	33	1a	22.66	44	2	0	1	2	NR
28	F	32	1a	1.16	60	2	0	0	5	NR
29	F	39	1a	1.47	51	6	3	1	2	NR
30	F	31	1a	11.55	41	6	1	2	2	NR
31	F	29	1a	1.03	35	4	1	1	3	NR
32	M	32	1a	0.27	141	6	1	8	2	NR
33	M	26	1a	12.27	99	5	2	3	2	BT
34	M	28	1a	18.79	116	5	1	0	2	BT
35	M	28	1a	6.21	131	6	0	1	1	BT

*GE/mL, genome equivalents per millilitre; ALT, alanine aminotransferase; aa, amino acid; N/D, not done; NR, nonresponder; REL, relapser; CSR, complete sustained responder; BT, breakthrough.
[a]For definitions of response, see text.

RESULTS

Response to Treatment and Relationship to Patients' Characteristics, Pretreatment Alanine Aminotransferase Levels, Liver Histologic Features, Genotype, and Viral Load

Thirty-seven patients with chronic HCV infection were enrolled in the study, but two were withdrawn because they did not complete the course of IFN-α. Patients' characteristics, pretreatment ALT levels, liver histologic features, genotype, and viral load are shown in Table I. Of 35 patients (19 men and 16 women with a mean age of 37.9 years; range, 25–58 years) who completed IFN-α therapy per the protocol, 17 (49%) were NRs, six (17%) had a BT of HCV RNA before completion of 48 weeks of treatment, six (17%) were deemed Rels at the end of treatment, and six (17%) were CSRs 6 months after treatment.

A difference in response rate to IFN-α was seen with different genotypes of HCV. Three of eight (38%), three of 13 (23%), and 12 of 14 (86%) patients infected with HCV types 1b, 1a, and 3a, respectively, showed negative results for HCV RNA at week 12 (chi-square test, $P = 0.003$). By 6 months after treatment, a complete sustained response was seen in two of eight (25%), none of 13 (0%), and four of 14 (29%), but the differences were not significant. No significant difference between response at either 12 or 72 weeks was seen in relation to sex, age, pretreatment ALT level, viral load, or Ishak score for inflammation or fibrosis.

Lack of Correlation Between the Interferon Sensitivity-determining Region Amino Acid Sequence Pretreatment Samples and Outcome of Treatment

Figure 1 shows an alignment of the pretreatment sequences of the 35 patients in the region equivalent to HCV-J [Kato et al., 1990] aa 2178 to 2390 according to genotype and response. The consensus aa sequence for each genotype is also shown. Considering the ISDR as originally reported (aa 2209-2248), the consensus at 12 weeks of this cohort of eight HCV subtype 1b isolates differed from the prototype IFN-resistant ISDR [Eno-

moto et al., 1995] at position 2218 (arginine to histidine), and pretreatment and 12-week consensus sequences were identical. Only one patient, P6, had an ISDR identical to the prototype IFN-resistant ISDR, though this patient had an initial response but relapsed off treatment. Amino acid differences at up to nine positions were seen, with a 3 aa insertion in patient P3. The most common aa difference was found at codon 2218, which nonetheless did not distinguish IFN-resistant from -sensitive sequences. Two NR sequences, P4 and P3, differ from the 12-week consensus by five and nine aa, respectively, the latter also having a 3 aa insertion at codon 2214. The 26 aa downstream of the ISDR were then included in the analysis, but no pattern of aa difference could be discerned to discriminate responders from NRs. Where there were aa differences from the consensus, the overall charge was the same or differed by only one fewer basic (P4) or acidic (P7) residue.

It was hypothesised that the consensus sequence obtained from NRs at week 12 of IFN-α treatment would be the IFN-resistant phenotype of the HCV type 1a samples. This sequence differed at only aa 2217 (arginine to threonine) from the prototype 1a sequence [Choo et al., 1991]. Alignment of sequences from 13 type 1a–infected patients showed that there were 12 variable residues in the ISDR. Differences in zero to eight aa positions were found, compared with the 12-week consensus. The most common aa differences were threonine to alanine (hydroxyl to aliphatic residue) at position 2217 and glutamic acid to alanine (acidic to aliphatic) at position 2236, seen in three and five of 13 patients, respectively. Of 10 NRs, only one, P26, was found to have the 12-week consensus sequence pretreatment; all other NRs had either one (P5) or two (P3) aa differences, except for patient P32, who had eight aa differences. Three patients who experienced an initial response to IFN had zero, one, and three aa differences from the 12-week consensus. Further analysis of the 26 aa downstream region was not carried out, since there were no CSRs.

The most striking feature of the alignment of type 3a ISDR aa sequences obtained from pretreatment serum samples is the lack of variability between isolates compared with the equivalent region of genotype 1. All but two sequences were identical to the 12-week consensus and the prototype sequence NZL1 [Sakamoto et al., 1994], with only six variable residues in the ISDR. Patient P18, whose results remained RT-PCR negative throughout treatment but who relapsed by week 72, had four aa differences from the consensus. Patient P22 was a CSR with a pretreatment sequence with 3 aa differences. When the 26 aa downstream of the ISDR are included in the analysis, another three sequences have differences from the consensus, and all of them are from either CSRs or Rels. In all five isolates with aa differences from the consensus, the overall charge was the same or differed by only two fewer basic residues (P18) or one additional acidic residue (P22).

Comparison of Pretreatment and 12-week Nonresponders

HCV in the serum exists as a swarm of quasi-species [Martell et al., 1992]. Failure to verify a relationship between the ISDR sequence of the pretreatment sample and ultimate response to interferon may stem from the fact that the IFN-α-resistant quasi-species is present as only a minor species that then becomes dominant in response to therapy. If the new dominant sequence were to be selected on the basis of aa differences, the ratio of synonymous to nonsynonymous differences (Ks/Ka) would be expected to be low. To address this issue, pretreatment sequences from 15 NRs (four of type 1b, nine of type 1a, and two of type 3a) were compared with those taken at 12 weeks. Only a single aa difference (nucleotide 2218, arginine for histidine) was seen in the ISDR as originally reported. Table II shows that, considering the nucleotides equivalent to those from 2209 to 2281 of HCV-J, the predominant sequence changed after 12 weeks of interferon therapy in nine of 15 patients, but they resulted in an aa change in only four of 15. In three of the four cases there were more synonymous than nonsynomous nucleotide changes, as shown by the Ks/Ka ratio. In the fourth case, there was a single nucleotide change, which was nonsynonymous. In patients from whom we had appropriately paired sequences in the region equivalent to HCV-J aa 2282-2385, which includes the previously reported V3 region [Inchauspe et al., 1991] and potential nuclear localisation signal [Ide et al., 1996], five of 12 had nucleotide differences. In only two, however, were there aa changes, and in both cases synonymous outnumbered nonsynonymous changes. These data do not provide support for selection in these regions of the NS5a gene on the basis of aa sequence.

Single-stranded Conformation Polymorphism

Direct sequencing of PCR products derived from serum will give the nucleotide present at each position in the majority of variants within a quasi-species. The consensus sequence over several hundred bases, however, may not represent that of any single variant; for this reason, emergence of a new majority sequence may be masked, particularly if there are a large number of variants and the majority forms less than 50% of the total. SSCP detects the number of variants in the region examined by differences in mobility of the individual strands of cDNA, which depend upon the nucleotide sequence of the whole length. SSCP was carried out on all pretreatment samples and those obtained at 12 weeks from NRs. The PCR products contain the nucleotides coding for the additional downstream aa identified as interacting with IFN-induced PKR [Gale et al., 1998] as well as the ISDR. The sensitivity of SSCP was established using as template five clones differing by four to 33 nucleotides, which were obtained by ligation into the *Sma*1 site of the pUC119 region of PCR products containing the hypervariable region of the E2 gene (nucleotides 1296-1646) [Choo et al., 1991]

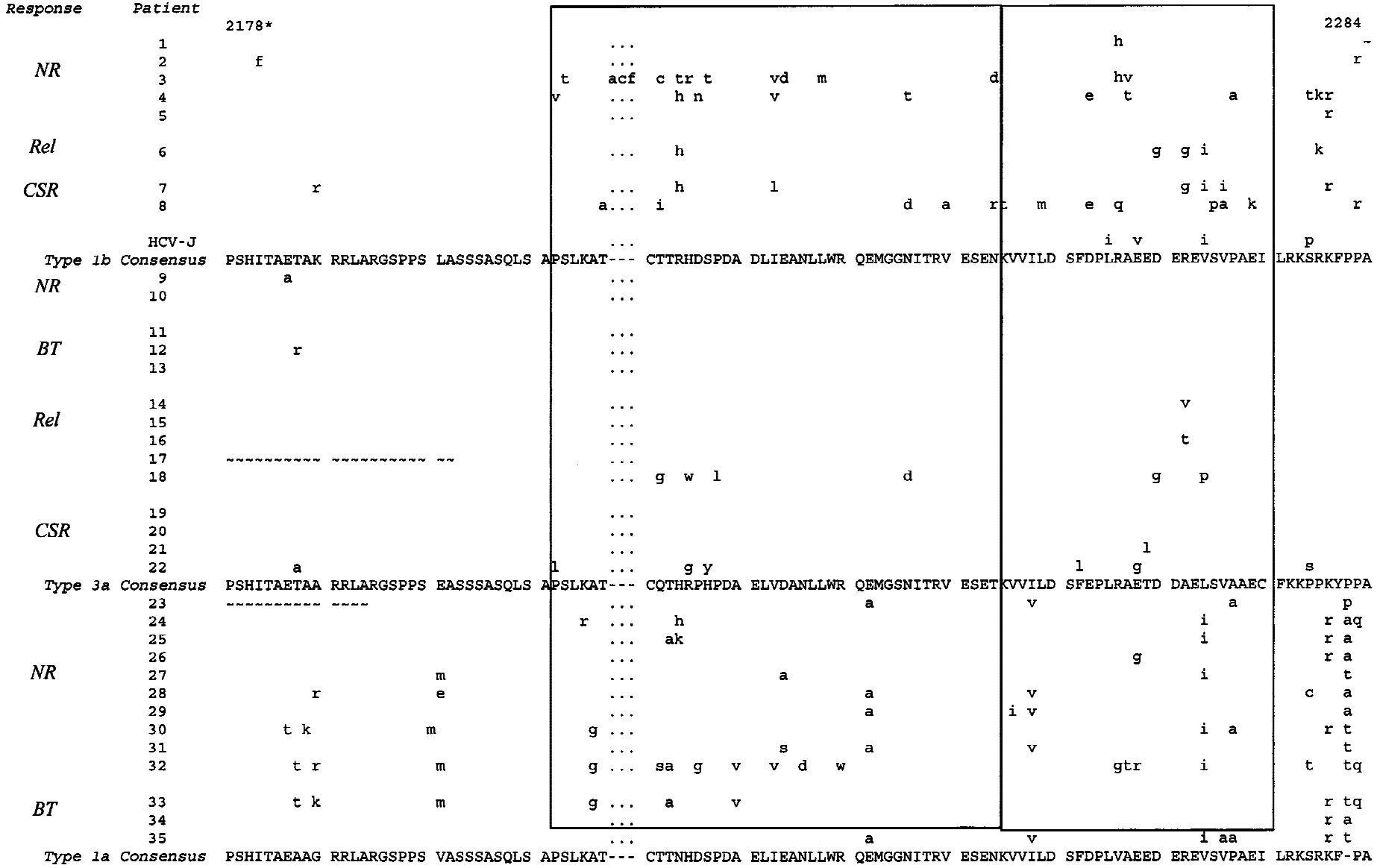

Fig. 1. Alignment of pretreatment derived amino acid sequences from part of the NS5a gene from 35 patients treated with interferon-α. Amino acid numbering is according to HCV-J [Kato et al., 1990]. The putative ISDR [Enomoto et al., 1995] is boxed in dotted lines, as are 31 amino acids downstream reported to interact with PKR [Gale et al., 1998]. ~ symbols indicate that the sequence was not obtained; dots denote amino acids not present. NR, nonresponder; Rel, relapser; BT, breakthrough; CSR, complete sustained responder. For definitions, see text. The consensus sequences obtained at week 12 for that genotype in nonresponders are shown.

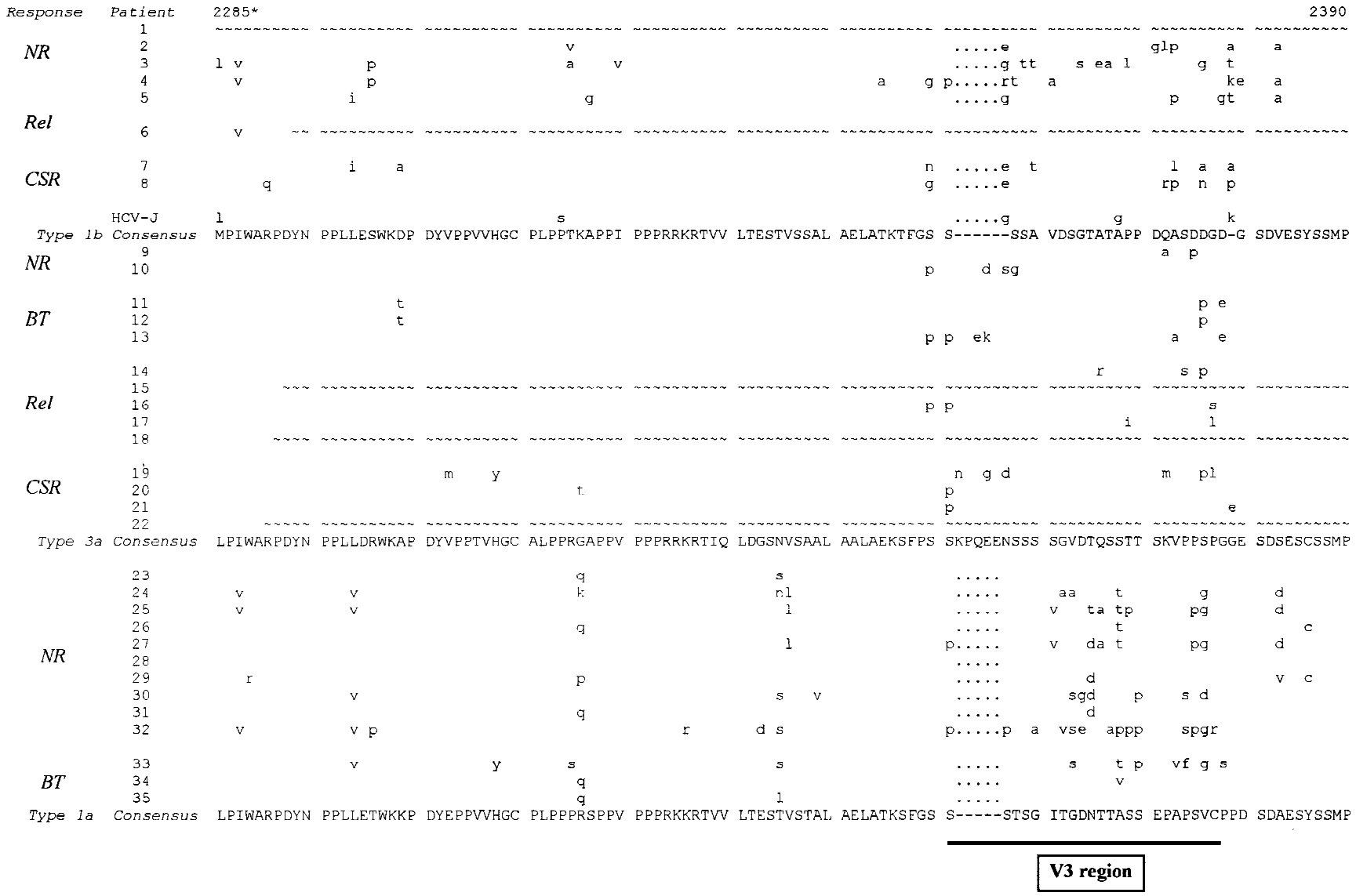

Figure 1 (continued).

TABLE II. Comparison of Nucleotide and Amino Acid Sequences at 3′ End of NS5a From Serum Samples Taken Before and at 12 Weeks Interferon Therapy

		Amino acids 2209–2281[a]			Amino acids 2282–2385[a]			Variation in SSCP profile between wk 0 and wk 12		
Patient	Genotype	Nt differences	aa changes (position)[a]	Ks/Ka	Nt differences	aa changes (position)[a]	Ks/Ka	Change in profile	Change in dominant band	No. of aa differences from "resistant" ISDR[b]
1	1b	3	h to k (2260) N	1.15	ND	ND	ND	Yes	Yes	0
2	1b	0	None	n/r	0	0	n/r	Yes[c]	Yes[c]	0
3	1b	1	None	n/r	0	0	n/r	Yes	Yes	12
4	1b	0	None	n/r	0	0	n/r	Yes	Yes	5
9	3a	0	None	n/r	4	0	n/r	No	No	0
10	3a	0	None	n/r	2	0	n/r	No	No	0
24	1a	0	None	n/r	20	k to r (2302) n to t (2338) l to v (2339) a to t (2365)	5.34	No	No	2
25	1a	0	None	n/r	5	s to t (2319) t to n (2369)	2.26	No	No	2
26	1a	2	g to r (2262)	0.001	1	0	n/r	Yes	Yes	0
27	1a	1	None	n/r	0	0	n/r	Yes	Yes	1
28	1a	14	a to m (2236) v to i (2251) i to v (2268) c to s (2278)	6.09	0	0	n/r	Yes	Yes	1
29	1a	6	i to v (2250)	2.95	ND	ND	ND	Yes	Yes	1
30	1a	4	None	n/r	0	0	n/r	Yes	Yes	1
31	1a	1	None	n/r	ND	ND	ND	No	No	2
32	1a	1	None	n/r	0	0	n/r	ND	ND	8

Nt, nucleotide; aa, amino acid; Ks/Ka, synonymous/nonsynonymous differences; ISDR, interferon sensitivity-determining region; n/r, not relevant; ND, not done.
[a]Amino acid numbering equivalent to HCV-J.
[b]HCV-J or 12-wk consensus.
[c]Differences in Nt sequence outside region analysed but within polymerase chain reaction product.

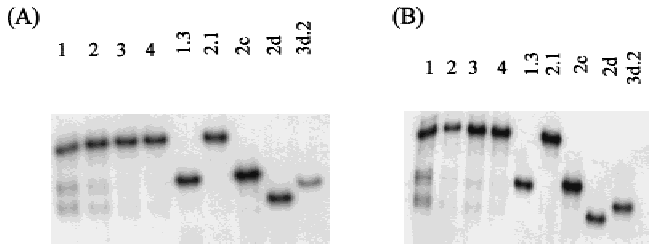

Fig. 2. Sensitivity and reproducibility of single-stranded conformation polymorphism (SSCP) analysis. Autoradiograph of SSCP gels run on different days under the same conditions. Independently generated single-stranded DNA products were used in each. **A**: 31.10.96; **B**: 4.11.96. Clones 2d, 1.3, 2.1, 2c, and 3d.2 were mixed at various proportions before polymerase chain reaction. Lanes 1–4: Clone 2.1 was present at 60%, 80%, 92%, and 96%, respectively, with the remaining clones at 10%, 5%, 2%, and 1% of the total. Lanes 5–9: individual clones as labelled.

derived from a patient's serum. To simulate an HCV quasi-species, one major and four minor variants were mixed at various ratios (10:10 ng to 10:0.05 ng per millilitre), and SSCP sensitivity was determined. All minor bands could be seen when present as 2% of the original template mixture. Electrophoresis at room temperature overnight showed greater separation and sharpness than at 4°C and was reproducible such that autoradiographs from repeated experiments on different days could be superimposed (Fig. 2). Alternative methods of generation of single-stranded DNA shown to be less satisfactory included asymmetric PCR using two primers, one at various limiting primer concentrations; PCR for 30 and 50 cycles; and use of *Pfu Taq* polymerase, which has proof reading activity.

Each patient was found to have a reproducible pretreatment SSCP profile with one to five bands, with a mean of 2.54 ± 0.2 (Table I). Ethidium bromide–stained gels and autoradiographs gave essentially similar results, though in some cases there was lack of clarity on autoradiographs owing to uneven uptake of radiolabel. Overall, there were one to five bands (mean 2.53 ± 0.29) in NRs, one to four bands (mean 2.56 ± 0.27) in responders at 12 weeks, and one to five bands (mean 3.17 ± 0.5) in CSRs. There was no significant difference in the number of SSCP bands pretreatment between 12-week responders and NRs or between CSRs and NRs (Student *t* test) nor between genotypes (one-way analysis of variance).

To determine whether a change in SSCP pattern occurs in the absence of exogenously administered IFN-α, SSCP was carried out on PCR products amplified from serum samples taken from 12 to 28 weeks apart, before treatment, from 10 patients. The pattern was compared with that obtained from serum immediately before treatment. A change in the dominant sequence was seen in six of 10 (60%), and a change in overall profile without change of dominant sequence was found in one of the remaining four. The analysis then was carried out on pretreatment and week 12 samples from 14 NRs. A change in dominant quasi-species variant by SSCP after 12 weeks of IFN-α therapy was seen in eight of 14 (57%). A change in the dominant quasi-species by SSCP always correlated with a change in the majority sequence obtained by direct sequencing. It is concluded that even in the absence of endogenously administered interferon, a change of dominant variant within the quasi-species is common and that changes are not seen more often in patients on treatment. This conclusion provides evidence against selection for aa change in this region of the NS5a protein by therapeutically administered IFN-α.

DISCUSSION

Despite intensive searching, we could find no evidence in these patients that IFN-α therapy exerts any selective pressure on the aa sequence of the carboxy half of the NS5a protein, including ISDR—either on the dominant variants of the quasi-species or within minor subpopulations. Although type 3–infected patients responded well initially, overall the complete sustained response rate was equivalent to that of type 1b–infected patients. Type 1a–infected patients had a particularly disappointing response, with no CSRs among 15 patients treated. Since this was a prospective study, there was no bias in the selection of patients, and this result could not have been predicted.

Using the ISDR as originally defined (aa 2209-2248 of HCV-J) [Enomoto et al., 1996], there was clear evidence that "Enomoto's rule" could not be used as a clinical tool to predict response in the eight Scottish patients infected with genotype 1b. Furthermore, two patients with four and 12 aa differences, respectively, from HCV-J in the ISDR were NRs, and the only one with the wild-type sequence was a responder who relapsed after treatment finished. The eight isolates were highly variable, however, and this variability extended into the 26 aa downstream reported as interacting with IFN-α-induced PKR [Gale et al., 1998], the one wild type in the ISDR having two differences from the prototype in the latter region. One isolate from an NR with 12 differences from wild type had a three aa insertion. It may be that sequences that are very different from the wild type are nevertheless able to inhibit PKR. Overall, doses of IFN-α used and response rates to treatment were similar to those reported elsewhere [Enomoto et al., 1996], so such differences cannot explain the lack of correlation of response with the aa sequence in the ISDR. Similar considerations may apply to the 1a isolates, which also show quite a degree of variability from the consensus—although only one of nine NRs had more than two differences from the type 1a consensus in the ISDR as originally defined. Further analysis of strains of this genotype is precluded owing to the lack of responders in our study. Another possible confounding factor could have been lack of compliance with therapy. No mention was made in the original report concerning how this was ascertained [Enomoto et al., 1996], and there is no biochemical test that can be used to monitor whether interferon is actually taken. We believe compliance in our patients was good, because a single specialist nurse supervised

treatment and her records agree with those of the hospital pharmacy to which all patients returned used and unused vials of drug monthly.

If the extra 31 aa downstream of the ISDR are considered in the context of genotype 3a isolates, two of four patients with complete sustained response and three of five who relapsed following treatment have differences from the consensus, whereas all patients who did not respond or who had a BT while on treatment had the consensus sequence. This finding suggests that these strains are less able to inhibit PKR. IFN-α has many effects on cells, only one of which is to induce PKR. Consequently, it would seem unlikely that the interaction of PKR and NS5a would be the only determinant of IFN-α response, explaining why some patients with isolates that might be expected to inhibit PKR nevertheless cleared the virus. An interaction of the E2 gene of HCV with PKR has been reported [Taylor et al., 1999], and evidence that NS5a may interfere with alternative interferon-induced proteins was verified by the ability of a construct expressing an "interferon resistant" but not an "interferon sensitive" NS5a to inhibit encephalomyocarditis virus (EMCV) but not vesicular stomatitis virus (VSV). This finding suggests an effect on the 2′–5′ A synthetase pathway [Song et al., 1999]. Other investigators have reported on the relative conservation of aa sequence in the NS5a of genotype 3 HCV, suggesting the absence of an ISDR. In neither study were the downstream aa studied [Squadrito et al., 1997; Saiz et al., 1998]. In this study, however, this finding could reflect a greater conservation between isolates of this genotype in the west of Scotland when the variation of downstream regions is also compared with those of genotypes 1a and 1b. The levels of variation may reflect the amount of time that these infecting genotypes have been present with genotype 1b, as in other parts of Europe, being the first to become established, with type 3a arriving more recently and being disseminated by sharing of needles for intravenous drug use.

It is interesting that the most variable aa in the ISDR between patients pretreatment was at position 2217 because other authors have suggested that interferon treatment selects for aa change at this site [Pawlotsky et al., 1998]. In our series, however, no isolate differed at this position between pretreatment and 12 week samples in the same patient. Throughout the sequences analysed pretreatment and at 12 weeks, only random changes of aa were observed; there was no evidence for selection, as judged by the ratio of synonymous to nonsynonymous nucleotide change.

With type 1a–infected individuals, aa changes between 0 and 12 weeks occurred either upstream or downstream of relatively conserved motif WARDPYNDLL (aas 2288-2298) regions, but not at both locations. This finding indicates that there may be interactions with two different molecules, selection of strains that inhibit PKR being observed as variation in aa upstream of WARDPYNDLL and that occurring downstream reflecting interaction with a different cellular or viral protein. Another group [Duverlie et al., 1998] has suggested that the V3 region of genotype 1b, which is included in this study in the downstream region, undergoes selection in response to interferon and that the motif GSSESSAV (aa 2353-2360) occurs more frequently in NRs [Inchauspe et al., 1991]. Only one of our genotype 1b isolates had this motif, and it was present both pretreatment and at 12 weeks. No other isolate pair showed evidence of selection within this region.

In this study, great care was taken to circumvent potential generation of artefacts in the generation of the majority sequence. All SSCP analyses were carried out in duplicate on different days on PCR products generated from separate aliquots of serum. On electrophoresis, mobilities of corresponding bands were exactly the same, establishing that random errors, such as those by *Taq* polymerase, were not contributing to differences in observed nucleotide sequence. A standard quantity of 25 ng of amplified DNA was analysed, rather than a fixed volume of PCR product, since the chance of detecting minor species increases as the amount of amplified DNA is analysed [Gonzalez-Peralta et al., 1996]. The rigorous standardisation and determination of the limit of detection of minor subpopulations by SSCP, as done in our study, have rarely been established. Some researchers did not take into account the sensitivity of their method or whether it was reliable and reproducible [Fujii et al., 1996; Harada et al., 1996; Toyoda et al., 1997]. Other researchers verified the sensitivity of SSCP by comparing gel profiles with the number of quasi-species determined by the cloning and sequencing of PCR products [Shindo et al., 1996; Peters et al., 1997] or by mixing two plasmids with known differences in SSCP mobility at various ratios [Enomoto et al., 1994; Moribe et al., 1995].

Results presented in this study indicate that the pretreatment quasi-species complexity of the 400-bp region of the NS5a gene analysed cannot be used to predict response to IFN-α therapy. We showed that change in the NS5a quasi-species is surprisingly common during the normal course of infection, in the absence of exogenously administered IFN-α. Rapid change of quasi-species may indicate a healthy immune response with a greater likelihood of viral clearance. Four pretreatment pairs of samples were from partial or complete responders, four were from patients who did not later receive treatment, and only one was from a patient who was an NR when given IFN-α. To test this hypothesis further, it would be necessary to study the rate of change in SSCP profile in the same patients before and during treatment. Two recent publications have examined the quasi-species heterogeneity of the ISDR by SSCP or by a similar technique, known as heteroduplex gel shift analysis (GSA). NS5a PCR products were first cloned into a plasmid vector rather than being examined directly, as in our study. The region of interest was then amplified from the individual clones and subjected to GSA or SSCP. The first study found no significant change in the ISDR quasi-species distribution before and after IFN-α treatment using GSA [Hof-

gartner et al., 1997]. The second, using SSCP, showed that the NS5a gene of HCV type 1b has a quasi-species distribution and that evolution of NS5a quasi-species during and after IFN-α treatment did occur. This evolution, however, was due to random change, except for aa 2217 and 2218; here there was a high nonsynonymous to synonymous ratio of nucleotide change, suggesting selection for aa change [Pawlotsky et al., 1998]. Neither study included control patients who had not undergone IFN-α treatment.

In conclusion, it is clear that no specific aa sequence in the ISDR, either as originally defined or as expanded to include the downstream 26 aa that interact with PKR, could be used accurately to predict which patients will respond to IFN-α therapy. Some findings, however, indicated that this region has an influence on response, and other potential interactions of HCV proteins with interferon-induced proteins have recently described [Song et al., 1999; Taylor et al., 1999]. Response in an individual probably depends on the balance of these interactions. Genotype 3 isolates were more conserved than genotypes 1a and 1b. Evidence suggesting that genotype 3a isolates are more likely to respond to treatment if there are aa differences from the consensus was the finding of such differences only in CSRs or Rels. There was no evidence to support the notion that NS5a quasi-species complexity pretreatment predicts response to IFN-α. Changes of majority sequence and quasi-species profile by SSCP were common in both treated and untreated patients.

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